

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification<sup>6</sup> : <b>C11B 7/00, C11C 1/04, 1/08, 3/00, C12P 7/64, A23D 9/00, 7/00, A23L 1/24, A23G 9/02</b></p>	<p><b>A1</b></p>	<p>(11) International Publication Number: <b>WO 96/37586</b> (43) International Publication Date: 28 November 1996 (28.11.96)</p>
<p>(21) International Application Number: PCT/EP96/02132 (22) International Filing Date: 13 May 1996 (13.05.96) (30) Priority Data: 95303535.9 24 May 1995 (24.05.95) EP (34) Countries for which the regional or international application was filed: AT et al. (71) Applicant (for all designated States except US): LODERS CROKLAAN B.V. [NL/NL]; Zaandijkeweg 36, NL-1521 AX Wormerveer (NL). (72) Inventors; and (75) Inventors/Applicants (for US only): CAIN, Frederick, William [GB/NL]; Dr. Blookerstraat 12, NL-2271 VL Voorburg (NL). HARRIS, John, Bernard [GB/GB]; 20 Colworth Road, Sharnbrook, Bedford MK44 1ET (GB). MOORE, Stephen, Raymond [GB/GB]; 2 Wainwright Avenue, Thrapston, Northamptonshire NN14 4UH (GB). MCNEILL, Gerald, Patrick [GB/GB]; 32 Lynford Way, Rushden NN10 9LZ (GB).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: PRODUCTION METHOD FOR FATS WITH LONG CHAIN POLYUNSATURATED FATTY ACIDS</p> <p>(57) Abstract</p> <p>Materials, enriched in long chain poly-unsaturated fatty acids (= LCPUFA) can be obtained from a material A, containing at least 5 % LCPUFA's by splitting A in materials B and C, B having to different LCPUFA's, while its total content LCPUFA's is <math>\geq 1.5</math> times the total LCPUFA content of A; B is split further in components D and E, D being enriched in a particular LCPUFA (<math>L_1</math> or <math>L_2</math>) by a factor of <math>\geq 1.5</math>, compared to B and E being depleted in the same LCPUFA.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Cote d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

**Production method for fats with long chain polyunsaturated fatty acids.**

It is fairly known from the literature, that fats, having a minimum amount of long chain polyunsaturated fatty acids (=LCPUFA's) do have a number of health benefits. (cf. e.g. EP 265.699; WO 90/04012; WO 94/00044; European Patent Application 95302942.8; EP 298.293 etc.) Moreover it is known that these fats can also suitably be applied in infant-food formulations. However for above applications it would be very beneficial if fats could be obtained, that have increased levels of LCPUFA's and/or wherein specific ratios between different LCPUFA's (e.g. L<sub>1</sub> and L<sub>2</sub>) present in the fat could be achieved, as different LCPUFA's, such as DHA and EPA have a different health effect. In particular it would be very beneficial if fats could be made with high levels of EPA, as it is very difficult to make these fats by conventional one step processes. It would be most suitable, if such fats could be obtained from cheap fat sources without having to apply complicated and expensive chemical and/or physical conversion-methods. It would also be very advantageous, if fats made according to such methods could be used for the preparation of concentrates, wherein the LCPUFA's would be present in specific levels and ratios, so that these concentrates could be blended with other fats with minimal changes in their functional properties.

Shimada discloses in J Am Oil Chem. Soc. 71 (1994) 951-954 a process for the concentration of DHA and EPA in glycerides by hydrolysing triglycerides, containing them with *Geotrichum candidum* or with *Candida cylindracea*. The hydrolysis treatment can be repeated with the same enzyme. However from the data mentioned in this paper it can be concluded that the enrichments achieved are too low for our aims.

Tanaka in J Am Oil Chem. Soc. 71 (1994) 331-334 discloses a process, wherein a fish oil is subjected to enzymic hydrolysis, using *Candida rugosa*, whereupon the total, crude mix obtained is subjected to directed titration. As a  
5 result free fatty acids are obtained with an unknown level of DHA and EPA, while also a glyceride-mix, containing mono;di-and tryglycerides, but also including some free fatty acids, is obtained. This total mix is reesterified, resulting in triglycerides with an increased DHA-level  
10 compared with the starting fish oil and with a slightly decreased EPA-level, compared with the starting fish oil. So the directed titration is performed on the total crude mix, resulting from the first enzymic conversion; therefore the results of the directed titration are insufficient and  
15 the method is uneconomic.

From JP 07/051075 a process is known, wherein a fish oil is subjected to hydrolysis in the presence of *Cand.cyl.* or *Cand.rugosa*. The resulting product has an increased DHA-  
20 level. This product is further hydrolysed, using a lipase from *Penicillium*. So by this treatment the diglycerides are removed from the mixture. The oil layer, resulting from the first hydrolysis can also be subjected to a basic ethanolic extraction. According to the data mentioned our aims for  
25 enrichment (ratio  $L_1:L_2$  and total  $L_1+L_2$ ) can not be achieved by this process.

According to JP 05/095792 a three step process is disclosed, wherein in a first step a hydrolysis of a fish  
30 oil is performed, using e.g. *Pseudomonas* lipase. The resulting product is concentrated in highly unsaturated fatty acids, e.g. by low temperature fractionation, urea adduction or absorption methods. The concentrate obtained is reconverted into triglycerides by reaction with  
35 glycerol, using e.g. genus *Candida*, while water is removed.

However the glycerides and free fatty acids, formed in the first step are not separated and therefore the second step is performed on the crude mixture obtained in the first step. This causes that enrichments obtained are  
5 insufficient.

In JP 90/071781 a process is disclosed, wherein a fish oil is split by treatment with *Cand. rugosa*. The resulting product is separated in free fatty acids and in glycerides.  
10 The free fatty acids are converted to esters by reaction with an alcohol, while the esters formed are subjected to urea adduction. The glycerides obtained by the separation are converted into esters by reaction with alcohol, where upon the esters formed are subjected to urea adduction.

15 According to J. Japan, Oil Chem. Soc. (1993), 35-43 a fish oil is hydrolysed in the presence of *Cand. cyl.* whereupon the mix obtained is subjected to an enzymic treatment for the removal of diglycerides. The free fatty acids obtained  
20 are converted to triglycerides. Although some enrichment in  $L_1$  plus  $L_2$  will be obtained, our levels of enrichments can not be achieved by this disclosed technology.

So far, our objectives could not be achieved by known  
25 preparation-methods. Therefore we studied whether we could find novel methods, with which above objectives could be fulfilled. This study has resulted in our invention. Basically our invention concerns a novel process for the production of materials, enriched in long chain poly-  
30 unsaturated fatty acids (= LCPUFA), wherein a material A, containing at least 5 wt% of total LCPUFA's is first split into two parts B and C; B having at least two different LCPUFA's, from which  $L_1$  and  $L_2$  are the two most abundant LCPUFA's, while B has a total LCPUFA-content that is at  
35 least 1.5 times greater than that of A; B is split into at least two components D and E, wherein D compared to B is

enriched by a factor of at least 1.5 in one of the LCPUFA's  $L_1$  or  $L_2$  and E simultaneously, compared to B, is depleted in the same LCPUFA  $L_1$  or  $L_2$ .

5 Material A thus contains at least 5 wt% of LCPUFA's, however higher levels of LCPUFA's in the end-product are obtained, when material A contains at least 10 wt%, preferably at least 15 wt%, more preferably at least 20 wt% and most preferably 25-50 wt% of LCPUFA's.

10

Very suitable materials A are selected from the group, consisting of at least one of the following oils:

- (1) marine oils, in particular Menhaden oil; cod liver oil; tuna oil; sardine oil; anchovy oil; herring oil; sand eel oil; or salmon oil.
- 15 (2) oils from microbial fermentation, in particular from a *Mortierella* species; *Penicillium*; *Phytium*; *Chlorella*; *Euglena*; *Porphyridium*; *Monodus* or *Nitzschia*.
- 20 (3) vegetable oils, in particular linseed oil, evening primrose oil, borage oil or blackcurrant seed oil.

In particular the fish oils are suitable sources, as a number of fish oils are cheap, while they still contain relatively high levels of LCPUFA's, which LCPUFA's consist in general of different LCPUFA's, such as DHA (docosahexaenoic acid:  $C_{22:6}$ ) and eicosapentaenoic acid (or EPA:  $C_{20:5}$ ).

30

Beneficial fats are obtained, when the long chain polyunsaturated fatty acids  $L_1$  and  $L_2$  are selected from fatty acids with at least 18 carbon atoms and at least 3 unsaturations, preferably  $C_{18:3}$ ,  $C_{20:4}$ ,  $C_{20:5}$ ,  $C_{22:5}$ , and  $C_{22:6}$ .

35 however also  $C_{18:4}$ ,  $C_{18:5}$ ,  $C_{20:3}$ ,  $C_{22:3}$ ,  $C_{24:3}$ ,  $C_{24:4}$ ,  $C_{24:5}$  and  $C_{24:6}$  can be applied.

Although material A can be subjected to the split, it is also possible to hydrolyse A first, preferably using randomising methods, such as by applying a non-specific lipase or a base, such as ethanolic potassium hydroxide.

5 The product A<sup>1</sup> obtained is rich in free fatty acids.

The split of material A or A<sup>1</sup> into parts B and C can be performed in a number of ways. Very suitably this split is performed either by:

10 (i) low temperature fractionation, in particular solvent fractionation, followed by filtration to remove stearin-fraction.

or

15 (ii) directed interesterification, both chemically using a base and enzymically, which interesterification is followed by removal of precipitated saturated triglycerides by filtration, either dry or in solvent.

or

20 (iii) glycerolysis, both chemically, using a base and enzymically, which glycerolysis is followed by removal of precipitated saturated partial glycerides by filtration, either dry or in solvent.

or

25 (iv) hydrolysis, using a lipase that is selective against CPUFA's over other fatty acids, followed by evaporation, or by extraction with aqueous alcohol, preferably methanol, or by treatment with an inorganic or organic absorbent, preferably basic alumina or :

30 (v) urea adduction, followed by filtration to remove stearin fraction,

or

(vi) directed titration, i.e. a solvent fractionation of metal salts of the free fatty acids, followed by

35

filtration to remove stearin fraction and reconversion back to free fatty acids by addition of a strong acid.

The low temperature fractionation (i) is performed at  
5 temperatures between -20 and -65°C, in particular between  
-25 and -60°C. Although a dry-fractionation is possible, we  
found that better results are obtained, if a wet-  
fractionation is performed. Solvents that can be applied  
for such a wet-fractionation are e.g. hexane, petroleum  
10 ether and acetone. However other solvents known for the  
wet-fractionation of fats can also be used. Suitable  
weight-ratios fats: solvent are 1:8 to 1:2, preferably: 1:6  
to 1:4. The oleine-fraction is normally obtained in a yield  
of 10-50 wt%. The oleine-fraction is the fraction enriched  
15 in LCPUFA's (so this is part B).

The directed interesterification (ii) can be performed by  
adding a base, such as Na-methylate to the mixture. The  
temperature applied will range from -5 to 80°C, in  
20 particular 10-50°C. Because of the presence of the base an  
interesterification of fatty acid moieties, bonded at the  
glycerol backbone will occur. This will result in the  
formation of all kinds of triglycerides, including  
triglycerides rich in saturated fatty acids (such as  
25 trisaturated triglycerides). These triglycerides, rich in  
saturated triglycerides will precipitate in the crude  
reaction mixture and will therefore direct the  
interesterification. At the end of the conversion the  
precipitate is separated from the other (liquid)  
30 triglycerides. This separation can be performed by any  
known suitable separation-technique for separating a liquid  
and a solid phase. The liquid phase is our part B, the  
solid phase is our part C.

The interesterification can also be performed as an enzymic  
35 interesterification. In that instance we prefer to use a  
lipase selected from Chromobacterium, Pseudomonas,

Rhizomucor, Humicola, Rhizopus or Candida. The enzymic interesterification (ii) is performed in the presence of a limited amount of water (i.e. up to 2 wt%). The conditions that can be applied are set out in e.g. GB 1,577,933. This  
5 assures that high levels of triglycerides are obtained, while the formation of extensive quantities of diglycerides is avoided.

Again the reaction can be directed by precipitation of the triglycerides, rich in saturated fatty acid moieties.

10

The glycerolysis (iii) also can be performed by using a base (e.g. Na-methylate) or by using an enzyme. Enzymes, that are known for glycerolysis-purposes, are disclosed in our earlier patent-application EP 94302325.9. The crude  
15 reaction product is a mixture of triglycerides and partial glycerides (most diglycerides), with a whole spectrum of fatty acid moieties in it. However the triglycerides and partial glycerides rich in saturated fatty acid moieties will precipitate in the crude reaction-mixture. This  
20 precipitation will direct the course of the glycerolysis, so that a product B, enriched in LCPUFA's can be separated from a product C, enriched in saturated fatty acids.

The hydrolysis (iv) is performed by using a lipase, that is  
25 selective against LCPUFA's over other fatty acids. Examples of such lipases are: Geotrichum candidum, Lipase G and Mucor Miehei.

The products B and C obtained in this way are a mixture of  
30 triglycerides and partial glycerides (as product B) and a mixture of free fatty acids (as product C). Because of the use of a lipase that is selective against LCPUFA's over other fatty acids, product B is enriched in LCPUFA, while the fatty acids from product C are enriched in the non-  
35 LCPUFA's.

So summarizing the above the products B and C obtained after completing the splits (i) to (iv) are respectively;

- different triglycerides for B and C (reactions i and ii)
- 5 - a mixture of triglycerides and partial glycerides, both for B and C, which mixture is different for B compared with the mixture for C. (reaction iii)
- a mixture of triglycerides and partial glycerides (for B) and a mixture of free fatty acids (for C) (reaction
- 10 iv).

In a following step product B is split into products D and E by performing an enzymic hydrolysis, using a lipase that can distinguish LCPUFA's of different chain length,

15 preferably by using *Candida rugosa*. Products D and E are separated by physical separation methods. Suitable methods are : evaporation, extraction with an aqueous alcohol preferably methanol and treatment with an inorganic or organic absorbent, preferably basic alumina. As a result of

20 the use of a lipase, that can distinguish LCPUFA's of different chain length, so e.g.  $C_{22:6}$  over  $C_{20:5}$ , a glyceride product D is obtained, wherein the two long chain polyunsaturated fatty acids  $L_1$  and  $L_2$ , originally present in product B in ratio  $X_B$ , are now present in another ratio

25  $X_D$ . E.g. when using *Candida rugosa* product D will have a ratio:

$$\frac{C_{22:6}}{C_{20:5}}$$

30 which is higher than the ratio of these fatty acids in product B. The opposite will account for product E.

It is also possible to perform our process in such away that product B mainly comprises free fatty acids. This

35 product B can be split into D and E by an enzymic conversion with glycerol, using an enzyme selective against LCPUFA. The resulting mixture comprises triglycerides and

partial glycerides, depleted in LCPUFA and free fatty acids, enriched in LCPUFA. This mixture can be separated by physical separation methods. These methods include:

- evaporation;
- 5 - extraction with an aqueous alcohol, preferably methanol
- or treatment with an inorganic or organic absorbent, preferably basic alumina.

The products D and/or E as obtained by the enzymic  
10 hydrolysis or esterification are used for different purposes. E.g. part of product D or E, being a mixture of triglycerides and partial glycerides enriched in  $L_1$  or  $L_2$ , is hydrolysed, resulting in a mixture comprising different free fatty acids and glycerol; the glycerol is removed from  
15 the mixture and remaining free fatty acids are reconverted with another part of product D or E, preferably in such a way that the reaction mixture has a stoichiometric composition.

20 However product D or E, comprising mainly free fatty acids, enriched in  $L_1$  or  $L_2$  can also be converted to triglycerides by esterification with glycerol or with partial glycerides, preferably in ratios corresponding with stoichiometric compositions.

25 It is however also possible that reaction product D or E, comprising partial glycerides and optionally also triglycerides is converted with a free fatty acid or mixture of free fatty acids, in particular comprising  
30 saturated and mono-unsaturated free fatty acids, to a triglyceride mixture.

As mentioned before the products, as obtainable by the different processes have many health-benefits. So it is  
35 possible to use these products per se in a number of consumer products. However the products often suffer from

oxygen-sensitivity. In order to improve this oxygen-sensitivity blends of materials are made, comprising a mixture of the products, as obtainable by the process of claims 1-14 and anti-oxidants, selected from the group of  
5 natural or synthetic tocopherols or other anti-oxidants, enzymes with anti-oxidant properties, such as Glucose oxidase, catalase, BHA, BHT, TBHQ, ascorbyl palmitate, propyl gallate, lecithin, catechins or flavenols.

10 According to another embodiment of our invention the fats as obtainable by the process according to the invention or its blends with anti-oxidants can also be mixed with other lipid materials that have a solid fat index at 5°C (N<sub>2</sub>: NMR-pulse, not stabilised) that is at least 5 units  
15 different from the N<sub>2</sub> of the fatty products, obtainable by the process of claims 1-14 or of the blend of claim 15. In this way fatblends can be obtained, that are appropriate for specific applications.

20 Part of our invention are also consumer products, such as food products, in particular spreads, cream alternatives, infant food, ice cream, mayonnaise, dressings, toppings etcetera, pharmaceutical products, skin-care products, such as lotions or skin-creams comprising a fatty component or a  
25 free fatty acid, wherein the fatty component or the free fatty acid comprises a product as obtainable by the process according to claims 1-14, or wherein the fatty component or free fatty acid comprises a blend according to claims 15-16.

30

According to a last embodiment our invention also concerns the use of materials, enriched in LCPUFA's, wherein the products, as obtainable by the process of claims 1-14 or wherein the blends according to claims 15-16 are used to  
35 improve the health benefits of consumer goods, such as food products or personal products.

**EXAMPLES:****EXAMPLE 1**

Three batches each consisting of 800g of refined Chilean fish oil containing 100 ppm of TBHQ as antioxidant were each dissolved in 3200g of acetone and cooled to -65°C. Stearin fractions were removed by filtration and washed with another 3200g of acetone. All the washes and oleine fractions were combined to produce a long chain poly-unsaturated (LCPUFA) enriched product, the composition of which is given in table 1.1. Fatty acid compositions were determined by fatty acid methyl ester gas chromatography (FAME GC) using the method given in AOCS Ce 1b-89, free fatty acid (FFA) contents were determined by titration against standard sodium hydroxide solution and are expressed as % oleic acid. Partial glyceride contents were determined by silica gel high performance liquid chromatography (HPLC) using an evaporative light scattering detector with 12, hydroxy iso-octane as an internal standard.

450g of the oleine fraction were mixed with 3.2g of *Candida rugosa* lipase dissolved in 650g of water and stirred at 25°C for 68 hours under a nitrogen blanket until 60% of the triglycerides had been hydrolysed to free fatty acid. The mixture was rapidly heated to 80°C to destroy the enzyme activity then the lipid layer was decanted off.

30

The hydrolysed reaction mixture was deacidified by extraction into aqueous methanol. 446g of hydrolysed products were extracted with 3L of methanol containing 2.5 % water at 55°C.

35

A triglyceride/ partial glyceride rich fraction and a free fatty acid rich fraction were thus obtained with compositions as given in table 1.1.

- 5 90g of the triglyceride/ partial glyceride mixture were vigorously stirred with an equal volume of water and with 1g of *Rhizomucor miehei* immobilised onto Duolite. The mixture was stirred under a nitrogen blanket at 35°C for 140 hours until the free fatty acid content was
- 10 approximately 20 %. The enzyme was removed by filtration and the free glycerol removed by water washing. 100 ppm of BHT were added. This hydrolysed partial glyceride fraction was re-esterified to triglyceride using *Rhizomucor miehei* immobilised onto Duolite as catalyst. 90g of the partial
- 15 glyceride mixture were mixed with 5g of *Rhizomucor miehei* at 55°C for 96 hours under vacuum. The enzyme was removed by filtration. The remaining free fatty acid in the triglyceride rich product was removed by treatment with basic alumina in hexane. 100 ppm
- 20 of BHT were added. The composition of the triglyceride rich fat is given in table 1.1.

- The free fatty acid rich fraction from the methanol extraction was esterified with glycerol to produce a
- 25 triglyceride rich fat. 10 g of the fatty acids were mixed with 1 g of glycerol and 0.6g of *Rhizomucor miehei* immobilised onto Duolite. The mixture was stirred, in an open glass vial at 55°C for 136 hours with nitrogen blowing across the surface. The composition of the triglyceride
- 30 rich fat is given in table 1.1.

TABLE 1.1 ANALYTICAL DATA EXAMPLE 1

	% TG	%DG	%FFA	20:5	22:6	TOTAL LCPUFA	YIELD	
5	FISH OIL	98	1.7	0	15.9	12.3	33.5	A
	LOW TEMPERATURE SOLVENT FRACTIONATION							
	STEARIN			12.8	11.2	30.2	77%	C
10	OLEINE			27	15.9	53.2	23%	B
	60% HYDROLYSIS OF OLEINE FRACTION USING C. RUGOSA LIPASE							
15	60% HYDROLYSIS PRODUCT	30	9	61	27.6	15.9	53.4	
	DEACIDIFICATION BY METHANOL EXTRACTION							
20	PARTIAL GLYCERIDE FRACTION	83	8	8	24.8	28.9	65.9	D
	FREE FATTY ACID FRACTION	0	9	91	28.7	11.2	49.1	E
25	RECOMBINATION TO TRIGLYCERIDES USING M. MIEHEI LIPASE							
	PARTIALLY HYDROLYSE PARTIAL GLYCERIDE FRACTION THEN RECOMBINE TO TG	89	2	9	24.9	29.6	66.1	
30	RECOMBINED FFA FRACTION	78	18	2	28.2	10.5	48.7	

35 A "RANCH STYLE" DRESSING was prepared using the LCPUFA enriched recombined partial glyceride fraction which was

compared to a reference dressing made with sunflower oil.  
The formulation for the dressing is given in table 1.2

TABLE 1.2 RANCH STYLE DRESSING FORMULATION

		Weight percent
5	Liquid oil	25
	Maltodextrin	20
	Dried Egg yolk	0.8
	Xanthum gum	0.4
	Vinegar	5
10	Water	48.8

The liquid oil for the reference was sunflower and for the LCPUFA containing product was 90/10 sunflower oil/ enriched product.

The water and maltodextrin were first blended using a homogeniser. The egg yolk, xanthum gum and vinegar were sequentially added whilst continuing to stir until complete mixing had occurred. At this stage the pH =3.25 .

The liquid oils were slowly added to the aqueous phase whilst homogenising. Mixing was continued until all the oil appeared to have been dispersed. The dressings were then transferred to sterile bottles.

25

The dressings were evaluated after 24 hours storage at ambient temperature. The viscosities of the samples were determined using a Brookfield Viscometer fitted with a number 4 spindle rotating at 10 rpm. The samples were contained in identical 200ml plastic bottles hence the viscosities are directly comparable with each other. For

each sample the average of three measurements was taken with the sample being allowed to relax for 1 minute between each 1 minute of shear.

The oil droplet size distribution was determined using a 5 Malvern Mastersizer fitted with a 45mm lens.

TABLE 1.3 RANCH DRESSINGS EVALUATION RESULTS EXAMPLE 1

OIL	VISCOSITY cP	SAUTER MEAN PARTICLE DIAMETER $\mu\text{m}$
REFERENCE	4387	2.46
LCPUFA PRODUCT	4527	2.52

AN ICECREAM was prepared using the LCPUFA enriched recombined partial glyceride fraction which was compared to a reference ice-cream made with sunflower oil. The ice creams were made according to the following recipe:

	wt%
Fat blend	10.0
Skimmed milk powder	10.0
Icing sugar	12.0
25 Corn syrup solids	4.0
Dextrose monohydrate	2.0
Sherex IC 9330®	0.6
Water	<u>61.4</u>
Total	100.0

Sherex IC 9330® is a product from Quest International and comprises mono- and diglycerides admixed with different stabilizers.

The fat blend for the reference was PO / Sunflower oil 90/10 and the fat blend according to the invention was 90/10 PO/LCPUFA product.

- 5 All ingredients except the water and the fat were mixed. Then the cold water was added to this mixture. This mixture was heated in a water bath till a temperature of 70°C. Then the fully liquid palm oil (= PO) was added to the mixture while "stirred" in the ultra-turrax. This emulsion was  
10 cooled in a water bath of 20°C. The emulsion was stirred in the ultra-turrax again. The batch ice cream machine was held for 24 hours at -28°C prior to use. The emulsion was placed in the batch ice cream machine and stirred for 15 minutes. The resulting ice cream was stored at -20°C for 24  
15 hours and then evaluated.

The viscosity of the ice cream emulsion, prior to freezing was measured. The overrun and hardness were determined. The viscosity was measured by using the Haake viscometer.

- 20 Hardness was measured by using a Stevens texture analyser with a 45° cone at a speed of 0.5 mm/second till a deepness of 2 mm.

TABLE 1.4 ICE-CREAM EVALUATION EXAMPLE 1

25

Sample	Overrun (%)	Hardness (gram)
Reference	16.3	458
LCPUFA PRODUCT	62.7	293

30

The viscosities of the emulsions were similar.

EXAMPLE 2

5 5Kg of refined Chilean fish oil containing 100 ppm of TBHQ as antioxidant were mixed with 250g of *Geotrichum candida* lipase dissolved in 5kg of pH7 phosphate buffer and stirred at 30°C for 48 hours under a nitrogen blanket. At this stage the free fatty acid content was 34 % .

10

The mixtures were rapidly heated to 90°C to destroy enzyme activity, washed with water then dried under vacuum. The free fatty acids were removed by evaporation at 190°C at a pressure of 0.02 to 0.04 m Bars and a flow rate of 40 to 15 50 ml/ min.

A triglyceride/ partial glyceride mixture was thus obtained with a composition as given in table 2.1. Analytical procedures were as described in example 1.

20

2.2Kg of the partial glyceride fraction were mixed with 13.2g of *Candida rugosa* lipase dissolved in 2.2Kg of pH7 phosphate buffer and stirred at 25°C for 70 hours under a nitrogen blanket until 60% of the oils had been hydrolysed 25 to free fatty acid .

The mixtures were rapidly heated to 90°C to destroy enzyme activity, washed with water then dried under vacuum. The free fatty acids were removed by evaporation at 190°C at 30 a pressure of 0.02 to 0.04 m Bars and a flow rate of 30 to 35 ml/ min.

A second triglyceride/ partial glyceride mixture was thus obtained with a composition as given in table 2.1. 35 252g of the second partial glyceride fraction were vigorously stirred with an equal volume of water and with

5.3g of *Rhizomucor miehei* immobilised onto Duolite. The mixture was stirred under a nitrogen blanket at 35°C for 48 hours until the free fatty acid content was approximately 55 %. The enzyme was removed by filtration and the free glycerol extracted by water washing. 100 ppm of BHT were added. This hydrolysed partial glyceride fraction was re-esterified to triglyceride using *Rhizomucor miehei* immobilised onto Duolite as catalyst. 153g of the partial glyceride mixture were mixed with 7.8 g of *Rhizomucor miehei* and 7.7g of glycerol at 55°C for 184 hours under vacuum. The enzyme was removed by filtration. The remaining free fatty acid and partial glycerides in the triglyceride rich product were removed by treatment with silica gel in hexane. 100 ppm of BHT were added. The composition of the triglyceride rich fat is given in table 2.1.

The second partial glyceride fraction (before partial hydrolysis) was re-esterified with a mixture of fatty acids produced by the random hydrolysis of sunflower oil. 9.8g of the partial glyceride fraction were mixed with 1.2 g of sunflower oil acids and 0.5g of *Rhizomucor miehei* immobilised onto Duolite. The mixture was stirred in an open glass vial at 55°C for 168 hours with nitrogen blowing across the surface. The composition of the triglyceride rich fat is given in table 2.1.

The free fatty acid rich fraction from the *Candida rugosa* hydrolysis was esterified with glycerol to form a triglyceride rich product. 9.7 g of the fatty acids were mixed with 1.1 g of glycerol and 0.5g of *Rhizomucor miehei* immobilised onto Duolite. The mixture was stirred in an open glass vial at 55°C for 212 hours with nitrogen blowing across the surface. The composition of the triglyceride rich fat is given in table 2.1.

TABLE 2.1 ANALYTICAL DATA EXAMPLE 2

	%TG	%DG	%FFA	20:5	22:6	TOTAL LCPUFA	
5							
10							
15							
20							
25							
30							

A SPREAD was prepared using the LCPUFA enriched triglyceride fraction which was compared to a reference spread made with sunflower oil. The spreads were made with the following formulation:

Fat Phase

	Fat Blend	40 %
	Hymono 7804	0.3 %
5	Colour (2% $\beta$ -carotene)	0.02 %
	Total	40.32 %

Aqueous Phase (to pH 5.1)

10	Water	56.44 %
	Skimmed Milk Powder	1.5 %
	Gelatin (270 bloom)	1.5 %
	Potassium Sorbate	0.15 %
15	Citric Acid Powder	0.07 %

All percentages on product basis.

20 The fat blend for the reference was 13% InEs, 87% SF.

For the LCPUFA product, the fat blend used was:-

	InEs	13%
	Sunflower	78%
25	LCPUFA	9%
	PRODUCT	

In Es = interesterified mix of hardened palm oil  
and hardened palm kernel olein.

2 kg of material was prepared and processed.

30

A micro-votator processing lines was set up as follows:-

Premix conditions - Stirrer Speed 60 rpm  
- Temperature 50°C

35

- pump - Proportioning pump set at 60% (30 g/min.).
- 5 A<sub>1</sub> conditions - Shaft speed 1000 rpm  
- Temperature set at 8°C
- C<sub>1</sub> conditions - Shaft speed 1000 rpm  
- Temperature set to 10°C
- 10 A<sub>2</sub> conditions - Shaft Speed 1000 rpm  
- Temperature set to 10°C
- C<sub>2</sub> conditions - Shaft speed 1000 rpm  
- Temperature set to 13°C
- 15

The aqueous phase was prepared by heating the required amount of water to approximately 80°C and then, using a silverson mixer, slowly mixing in the ingredients. The pH of the system was adjusted to 5.1 by adding 20% Lactic acid solution as required.

A premix was prepared by stirring the fat phase in the premix tank and then slowly adding in the aqueous phase. When addition was complete, the mix was stirred for a further 5 minutes before pumping through the line. When the process had stabilised (around 20 minutes), product was collected for storage and evaluation.

TABLE 2.2 TYPICAL PROCESS CONDITIONS FOR SPREAD PRODUCTION

Sample	A <sub>1</sub> Exit (°C)	C <sub>1</sub> Exit (°C)	A <sub>2</sub> Exit (°C)	C <sub>2</sub> Exit (°C)	Line Pressure (bar)
5 Reference	13.2	18.7	13.6	15.6	0.5 to 2
LCPUFA product	13.0	18.9	12.2	16.8	1.3 to 2.2

10

Very good oil continuous low fat spreads were produced using this system for both the reference and the LCPUFA product.

The spreads were evaluated after 5 days storage at 5°C and 15 20°C for hardness using a cone penetrometer, electrical conductivity and for the plasticity of the product by formation of a collar. The results are given in table 2.3

TABLE 2.3 SPREAD EVALUATIONS EXAMPLE 2

20

Sample	5°C			20°C		
	C- Value g/cm <sup>2</sup>	Conductiv ity μScm <sup>-1</sup>	Collar	C- value g/cm <sup>2</sup>	Conductiv ity μScm <sup>-1</sup>	Collar
Contro l	170	10 <sup>-5</sup>	I	190	10 <sup>-5</sup>	I
25 LCPUFA produc t	150	10 <sup>-5</sup>	I	130	10 <sup>-5</sup>	I

(Collar formation is scored on a scale of 1 to 6 . A collar of 1 shows that the product has little structure a score of 6 has a lot of structure and is butterlike.)

- 5 Both samples spread very easily on grease-proof paper, with no obvious signs of water loss.

A "RANCH STYLE" DRESSING was prepared using the LCPUFA enriched triglyceride fraction which was compared to a  
10 reference dressing made with sunflower oil. The formulation and method of production was as described in example 1.

TABLE 2. 4 RANCH DRESSINGS EVALUATION RESULTS EXAMPLE 2

5 OIL	VISCOSITY cP	SAUTER MEAN PARTICLE DIAMETER $\mu\text{m}$
	Reference	4387
	LCPUFA Product	4613

10 AN ICE-CREAM was prepared using the LCPUFA enriched recombined partial glyceride fraction which was compared to a reference ice-cream made with sunflower oil. The formulation and method of production was as described in example 1.

15

TABLE 2.5 ICE-CREAM EVALUATION EXAMPLE 2

20	Sample	Overrun (%)	Hardness (gram)
	Reference	16.3	458
	LCPUFA PRODUCT	57.1	342

The viscosities of the emulsions were similar.

25

30

EXAMPLE 3

100g of Chilean fish oil were hydrolysed to free fatty acids by refluxing with 23g of potassium hydroxide in 5 130mls of ethanol and 44mls of water for 1hour. The potassium salts were converted to free fatty acids by addition of hydrochloric acid and then extracted into hexane.

10 21g of the fatty acids were mixed with 100mls of 0.5M sodium hydroxide and 200mls of acetone. The mixture was stirred in a jacketed vessel with a scrape surface stirrer at 40°C for 30minutes then cooled at 1°C/min to 4°C at which temperature it was stirred for 1 hour. The 15 crystalline stearin fraction was removed by filtration and washed with a further 75mls of acetone. The sodium salts in the stearin and oleine fractions were converted back to free fatty acids by addition of hydrochloric acid and then extracted into hexane. The compositions of the fractions 20 are given in table 3. Analytical procedures were as described in example 1.

The fatty acids were esterified with glycerol to form a triglyceride rich fat. 2.7g of the fatty acids were mixed 25 with 0.4g of glycerol, 0.3g of water and 0.25g of *Candida rugosa* lipase immobilised onto Accurel. The mixture was stirred in an open glass vial at 35°C for 120 hours with nitrogen blowing across the surface. The resulting glyceride species were separated by thin layer 30 chromatography and the fatty acid compositions determined by FAME GC.

TABLE 3 ANALYTICAL DATA EXAMPLE 3

	%TG	%DG	%FFA	20:5	22:6	TOTAL LCPUF A	YIELD	
	FISH OIL	98	2	0	16.3	11.3	33.9	A
5	RANDOM HYDROLYSIS USING ETHANOLIC KOH CATALYST							
	RANDOM HYDROLYSIS PRODUCT	0	0	100	16.3	11.3	33.9	
	SOLVENT FRACTIONATION OF SODIUM SALTS							
10	STEARIN	0	0	100	6	5	13.4	53% C
	OLEINE	0	0	100	25	18	52.7	47% B
15	RECOMBINATION OF OLEINE ACIDS TO TRIGLYCERIDES USING C RUGOSA LIPASE							
	RECOMBINATION PRODUCT	25	25	37	25	18	52.7	
	SEPARATION							
	TRIGLYCERIDE FRACTION				24.9	4.1	35.7	E
	DIGLYCERIDE FRACTION				28.2	3.8	40.5	
20	MONOGLYCERIDE FRACTION				28.9	4.5	40.3	
	FREE FATTY ACID FRACTION				23.3	31.5	66.3	
								D

25

30 EXAMPLE 4

100g of Chilean fish oil were hydrolysed to free fatty acids by refluxing with 23g of potassium hydroxide in

130mls of ethanol and 44mls of water for 1hour. The potassium salts were converted to free fatty acids by addition of hydrochloric acid and then extracted into hexane.

5

41g of the fatty acids were added to 200g of urea mixed with 750mls of ethanol at 65°C in a jacketed vessel fitted with a scape surface stirrer. The mixture was stirred for 2 hours at 70°C then cooled at 1°C/min to 4°C at which

10 temperature it was held for 16 hours. The solid fraction was removed by filtration. The ethanol was removed from the oleine fraction and the urea salts converted back to free fatty acids by addition of hydrochloric acid and then extracted into hexane.

15

The fatty acids were esterified with glycerol to form a triglyceride rich fat. 5.0 g of the fatty acids were mixed with 0.5g of glycerol, 0.3g of water and 0.1g of *Candida rugosa* lipase immobilised onto Accurel. The mixture was

20 stirred in an open glass vial at 35°C for 40 hours with nitrogen blowing across the surface. The resulting glyceride species were separated by thin layer chromatography and the fatty acid compositions determined by FAME GC. Analytical procedures were as described in

25 example 1.

TABLE 4 ANALYTICAL DATA EXAMPLE 4

	%TG	%DG	%FFA	20:5	22:6	TOTAL LCPUF A	YIELD	
5	FISH OIL	98	2	0	15.4	11.5	33.9	A
	RANDOM HYDROLYSIS USING KOH CATALYST							
	RANDOM HYDROLYSIS PRODUCT	0	0	100	15.4	11.5	33.9	
10	UREA FRACTIONATION							
	STEARIN							
	OLEINE	0	0	100	36.0	23.4	69.9	41% B
	RECOMBINATION OF OLEINE ACIDS TO TRIGLYCERIDE USING C RUGOSA LIPASE							
15	RECOMBINATION PRODUCT	15	14	57	36.0	23.4	69.9	
20	SEPARATION							
	TRIGLYCERIDE FRACTION				34.3	8.7	58.1	E
	DIGLYCERIDE FRACTION				42.3	5.9	60.9	
25	EMONOGLYCERIDE FRACTION				45.4	9.6	67	
	FREE FATTY ACID FRACTION				30.6	36.2	78.9	D

### Claims

1. Process for the production of materials, enriched in long chain poly-unsaturated fatty acids (= LCPUFA), wherein a material A, containing at least 5 wt% of total LCPUFA's is first split into two parts B and C; B having at least two different LCPUFA's, from which  $L_1$  and  $L_2$  are the two most abundant LCPUFA's, while B has a total LCPUFA-content that is at least 1.5 times greater than that of A; B is split into at least two components D and E, wherein D compared to B is enriched by a factor of at least 1.5 in one of the LCPUFA's  $L_1$  or  $L_2$  and E simultaneously, compared to B, is depleted in the same LCPUFA  $L_1$  or  $L_2$ .
2. Process according to claim 1, wherein material A contains at least 10 wt%, preferably at least 15 wt%, more preferably at least 20 wt% and most preferably 25-50 wt% of LCPUFA's.
3. Process according to claim 1 or 2, wherein material A is selected from the group consisting of at least one of the following oils:
  - (1) marine oils, in particular Menhaden oil, cod liver oil, tuna oil, sardine oil and anchovy oil.
  - (2) oils from microbial fermentation, in particular from a *Mortierella* species.
  - (3) vegetable oils, in particular linseed oil, evening primrose oil, borage oil or blackcurrant seed oil.
4. Process according to claims 1-3, wherein the long chain poly-unsaturated fatty acids  $L_1$  and  $L_2$  are fatty acids with at least 18 carbon atoms and at least 3 unsaturations, preferably  $C_{18:3}$ ,  $C_{20:4}$ ,  $C_{20:5}$ ,  $C_{22:5}$  and  $C_{22:6}$ .

5. Process according to claims 1-4, wherein material A is optionally hydrolyzed, using an enzyme or a base, to a product A<sup>1</sup>, rich in free fatty acids, whereupon A or A<sup>1</sup> is split into B and C by:

(i) low temperature fractionation, in particular solvent fractionation, followed by filtration to remove the stearin fraction.

or

(ii) directed interesterification, both chemically using a base and enzymically, which interesterification is followed by removal of the precipitated saturated triglycerides by filtration either dry or in solvent.

or

(iii) glycerolysis, both chemically, using a base and enzymically, which glycerolysis is followed by removal of the precipitated saturated partial glycerides by filtration, either dry or in solvent.

or

(iv) hydrolysis, using a lipase that is selective against LCPUFA's over other fatty acids, followed by evaporation, or extraction with aqueous alcohol, preferably methanol, or by treatment with an absorbing inorganic or organic material, preferably basic alumina.

6. Process according to claims 1-5, wherein the split of A or A<sup>1</sup> into B and C is performed by:

(i) urea adduction, followed by filtration to remove stearin-fraction.

or

(ii) directed titration.

7. Process according to claim 5 (iv), wherein the lipase is selected from: *Geotrichum candidum*, Lipase G and *Mucor Miehei*.
8. Process according to claim 5, wherein the products B and C, obtained according to claim 5 (i) and 5 (ii) are both triglycerides; according to claim 5 (iii) are both triglycerides and/or partial glycerides and according to claim 5 (iv) are triglycerides and/or partial glycerides (= product B) and free fatty acids (= product C).
9. Process according to claims 1-8, wherein product B is split into products D and E by performing an enzymic hydrolysis, using a lipase that can distinguish LCPUFA's of different chain length, preferably by using *Candida rugosa*, followed by a physical separation of D from E by either evaporation, or extraction with aqueous alcohol, preferably methanol or treatment with an inorganic absorbent, preferably basic alumina.
10. Process according to claim 1, wherein a product B, comprising mainly free fatty acids is split into D and E by an enzymic conversion with glycerol, using an enzyme selective against long chain PUFA, resulting in a mixture of triglyceride, and particle glycerides depleted in long chain PUFA and free fatty acids enriched in LCPUFA, which mixture is separated by physical methods.
11. Process according to claim 11, wherein the physical separation method is selected from :
  - evaporation;

or

- extraction with aqueous alcohol, preferably methanol
  - or treatment with an inorganic or organic absorbent, preferably basic alumina.
12. Process according to claim 1, wherein part of product D or E, being a mixture of triglycerides and partial glycerides enriched in  $L_1$  or  $L_2$ , is hydrolysed, resulting in a mixture comprising different free fatty acids and glycerol; removing the glycerol from the mixture and reconvertng the remaining free fatty acids with another part of product D or E, preferably in such a way that the reaction mixture has a stoichiometric composition.
13. Process according to claim 1, wherein product D or E comprising mainly free fatty acids, enriched in  $L_1$  or  $L_2$  is converted to triglycerides by esterification with glycerol or with partial glycerides, preferably in ratios corresponding with stoichiometric compositions.
14. Process according to claim 1, wherein the reaction product D or E, comprising partial glycerides and optionally also triglycerides is converted with a free fatty acid or mixture of free fatty acids, in particular comprising saturated and mono-unsaturated free fatty acids, to a triglyceride mixture.
15. Blends of materials, comprising a mixture of the products, as obtainable by the process of claims 1-14 and anti-oxidants, selected from the group of natural or synthetic tocopherols or other anti-oxidants, enzymes with anti-oxidant properties, in particular Glucose oxidase and/or catalase, BHA, BHT or TBHQ

16. Blends of materials, comprising a mixture of the products, as obtainable by the process of claims 1-14 or the blends of claim 15 and other lipid materials that have a solid fat index at 5°C ( $N_s$ : NMR-pulse, not stabilised) that is at least 5 units different from the  $N_s$  of the fatty products, obtainable by the process of claims 1-14 or of the blend of claim 15.
17. Consumer products, such as food products, in particular spreads, cream alternatives, infant food, ice cream, mayonnaise, dressings, toppings etcetera, pharmaceutical products, skin-care products, such as lotions or skin-creams comprising a fatty component or a free fatty acid, wherein the fatty component or the free fatty acid comprises a product as obtainable by the process according to claims 1-14, or wherein the fatty component or free fatty acid comprises a blend according to claims 15-16.
18. Use of materials, enriched in LCPUFA's, wherein the products, as obtainable by the process of claims 1-14 or wherein the blends according to claims 15-16 are used to improve the health benefits of consumer goods, such as food products or personal products.

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 96/02132

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C11B7/00 C11C1/04 C11C1/08 C11C3/00 C12P7/64  
A23D9/00 A23D7/00 A23L1/24 A23G9/02

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C11B C11C C12P A23D A23L A61K C07C A23G

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE WPI Week 9517 Derwent Publications Ltd., London, GB; AN 95-127359 XP002013469 &amp; JP,A,07 051 075 (NIPPON OILS &amp; FATS CO LTD), 28 February 1995 cited in the application see abstract &amp; PATENT ABSTRACTS OF JAPAN vol. 950, no. 2 &amp; JP,A,07 051075 (NIPPON OIL &amp; FATS CO LTD), 28 February 1995, see abstract</p> <p style="text-align: center;">--- -/--</p>	1-18

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \* "A" document defining the general state of the art which is not considered to be of particular relevance
- \* "E" earlier document but published on or after the international filing date
- \* "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \* "O" document referring to an oral disclosure, use, exhibition or other means
- \* "P" document published prior to the international filing date but later than the priority date claimed

\* "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\* "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\* "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\* "Z" document member of the same patent family

Date of the actual completion of the international search

17 September 1996

Date of mailing of the international search report

03.10.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+31-70) 340-3016

Authorized officer

Dekeirel, M

# INTERNATIONAL SEARCH REPORT

Int. Application No  
PCT/EP 96/02132

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 17, no. 429 (C-1095), 10 August 1993 & JP,A,05 095792 (AGENCY OF IND SCIENCE & TECHNOLOGY), 20 April 1993, cited in the application see abstract & DATABASE WPI Week 9320 Derwent Publications Ltd., London, GB; AN 93-163591 & JP,A,05 095 792 (AGENCY OF IND SCI & TECHNOLOGY) , 20 April 1993 see abstract	1-18
X	--- JOURNAL OF THE AMERICAN OIL CHEMISTS' SOCIETY, vol. 71, no. 9, 1994, CHAMPAIGN US, pages 951-954, XP002013466 YUJI SHIMADA ET AL.: "Enrichment of polyunsaturated fatty acids with Geotrichum candidum lipase" cited in the application see page 952, column 2, paragraph 3 - page 953, column 2, paragraph 1	1-14
X	--- JOURNAL OF THE AMERICAN OIL CHEMISTS' SOCIETY, vol. 71, no. 3, 1994, CHAMPAIGN US, pages 331-334, XP002010220 YUKIHISA TANAKA ET AL.: "Synthesis of docosahexaenoic acid-rich triglyceride with immobilized Chromobacterium viscosum lipase" cited in the application see the whole document	1-16
X	--- JOURNAL OF THE JAPAN OIL CHEMISTS' SOCIETY, vol. 43, no. 1, 1994, JP, pages 39-43, XP002013467 YUKIHISA TANAKA ET AL.: "Synthesis of DHA-enriched triacylglycerol" cited in the application * page 39, Abstract *	1-16
	--- -/--	

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 96/02132

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE WPI Week 9010 Derwent Publications Ltd., London, GB; AN 90-071781 XP002013470 &amp; JP,A,02 025 447 (NIPPON OILS &amp; FATS CO LTD) , 26 January 1990 cited in the application see abstract &amp; PATENT ABSTRACTS OF JAPAN vol. 14, no. 176 (C-0707), 9 April 1990 &amp; JP,A,02 025447 (NIPPON OIL &amp; FATS CO LTD), 26 January 1990, see abstract</p>	1-16
A	<p>--- JOURNAL OF THE AMERICAN OIL CHEMISTS' SOCIETY, vol. 70, no. 10, 1993, CHAMPAIGN US, pages 1031-1034, XP002013473 YUKIHISA TANAKA ET AL.: "Triglyceride specificity of Candida cylindracea lipase: effect of docosahexaenoic acid on resistance of triglyceride to lipase" see page 1034, column 1, paragraph 3</p>	1-4
A	<p>--- PATENT ABSTRACTS OF JAPAN vol. 8, no. 156 (C-234), 19 July 1984 &amp; JP,A,59 059644 (KUREHA KAGAKU KOGYO KK), 5 April 1984, see abstract &amp; DATABASE WPI Week 8420 Derwent Publications Ltd., London, GB; AN 84-123454 &amp; JP,A,59 059 644 (KUREHA CHEM IND KK) , 5 April 1984 see abstract</p>	1-4
A	<p>--- JOURNAL OF THE AMERICAN OIL CHEMISTS' SOCIETY, vol. 69, no. 2, 1992, CHAMPAIGN US, pages 1210-1214, XP002013468 YUKIHISA TANAKA ET AL.: "Concentration of docosahexaenoic acid in glyceride by hydrolysis of fish oil with Candida cylindrycea lipase" see the whole document</p> <p style="text-align: center;">-----</p>	1-4